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PURIFICATION OF ALVEOLAR EPITHELIAL TYPE II CELLS FROM RAT LUNG--ETC(U)
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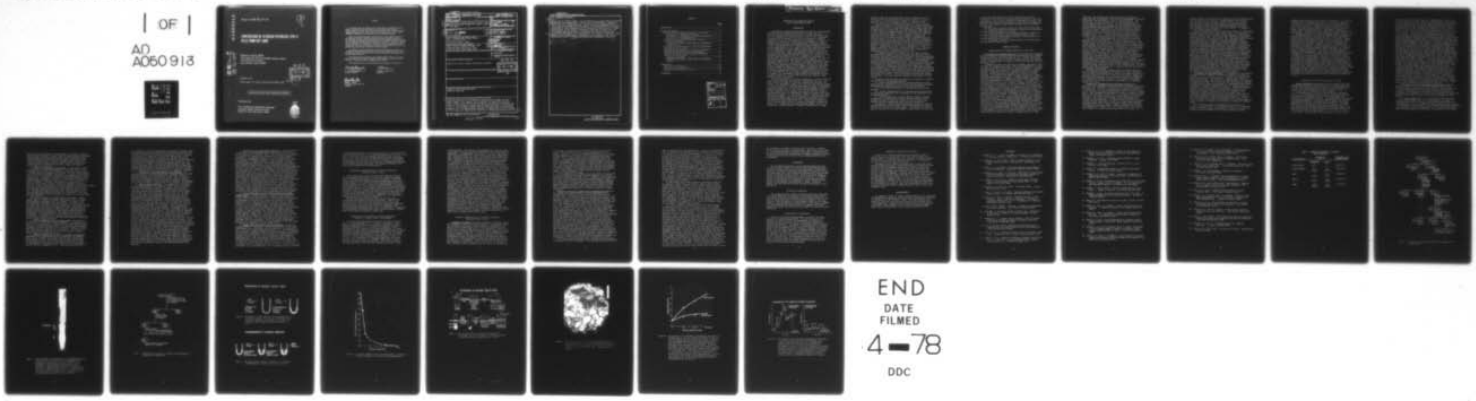

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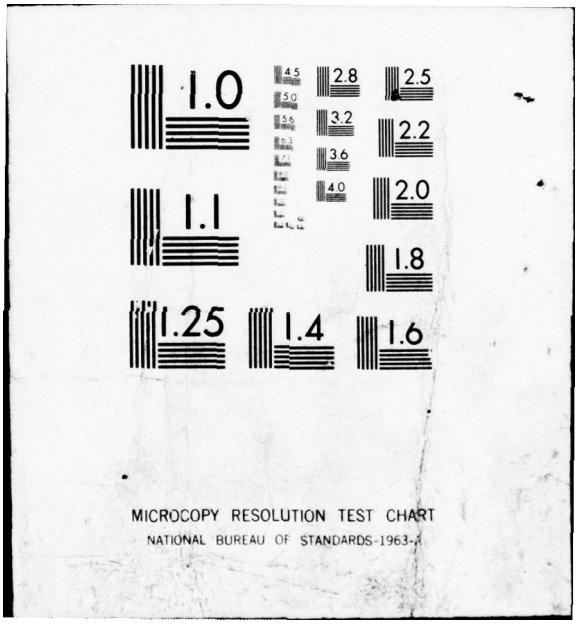
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Report SAM-TR-77-18

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PURIFICATION OF ALVEOLAR EPITHELIAL TYPE II CELLS FROM RAT LUNG

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December 1977

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Prepared for

USAF SCHOOL OF AEROSPACE MEDICINE
Aerospace Medical Division (AFSC)
Brooks Air Force Base, Texas 78235

NOTICES

This final report was submitted by The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284, under contract F41609-75-C0023, job order 2312-V5-04, with the USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, Brooks Air Force Base, Texas. Dr. F. Wesley Baumgardner (SAM/VNL) was the Laboratory Project Scientist-in-Charge.

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The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act of 1970 and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources - National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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Prolonged inhalation of above-ambient concentrations of oxygen can lead to severe damage of the lung and to the compromise of respiratory function. Several observations indicate that the surface properties of the material adsorbed at the alveolar air-liquid interface may be affected by such exposures. It is not clear, however, whether these perturbations result from the direct damage to the cells metabolizing surfactant, or whether they occur from damage to other lung cells resulting in an effusion of interfering materials into the → over

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20. ABSTRACT (continued)

alveoli and to alveolar edema. To help differentiate between these alternatives we reasoned that methods must be used by which the metabolism of pulmonary surfactant can be studied directly. These include: (1) the isolation of certain proteins associated with this material; (2) the purification of enriched populations of cells responsible for the synthesis of this material; (3) the quantification of the content of surfactant apoprotein in these cells; and (4) the investigation of some of the metabolic activities of these cells, particularly with regard to the metabolism of the proteins of surfactant. We have developed these necessary techniques with the support of this contract. The detailed results of these investigations are described in this report.

CONTENTS

PURIFICATION OF ALVEOLAR EPITHELIAL TYPE II CELLS FROM RAT LUNG

INTRODUCTION

Biochemical and morphological observations indicate that prolonged inhalation of above-ambient concentrations of oxygen can lead to structural damage to endothelial and Type I alveolar epithelial cells (20, 27), hyperplasia of alveolar Type II cells (1, 20), accumulation of interstitial and alveolar fluid (6, 16), deposition of hyaline membranes (6, 41), and interference with the formation or function of pulmonary surfactant (11). Physiological manifestations of these changes result in decreasing O_2 uptake because of impaired diffusion (5, 18), $V/\dot{Q}$ abnormalities (6), decreased pulmonary compliance (5), and alveolar atelectasis (6, 35). Despite this rather detailed description of the morphological and physiological changes resultant from the chronic inhalation of oxygen, the biochemical bases of these lesions are only speculative. Suggested mechanisms include the oxidation of glutathione and sulfhydryl-containing coenzymes (17), oxidation of the sulfhydryl groups of enzymes (8, 17), formation of oxidizing free radicals (10), increase in intracellular redox potential (37), and the interference with enzymes which neutralize free radicals, such as superoxide dismutase (7). The effects are probably mediated by several mechanisms and may vary among different cell types, even in the same organ. Thus, morphological studies suggest that the lung cells which are first damaged by oxygen are the pulmonary endothelial and Type I epithelial cells, and subsequently the Type II epithelial cells (20). No evidence suggests that the lesion is confined to any one cell type or is resultant from any one specific biochemical mechanism, and it is probable that above-ambient oxygen mixtures may compromise several biochemical functions, perhaps with differing time courses or with differing degrees of severity.

Among the biochemical activities carried out by alveolar epithelial cells is the elaboration and secretion of pulmonary surfactant. The direct effects of oxygen on surfactant synthesis and regulation are unknown. The experiments carried out to date have used indirect methods to assess the effects of breathing 100% oxygen at either one atmosphere or elevated pressures, and the results have been somewhat contradictory. Bondurant and Smith (2), in 1962, could find no difference in the surface properties of the tracheal foam obtained from rats killed by breathing 100% oxygen at 8 atmospheres pressure as compared with those breathing 12% oxygen. In later studies, however, this same group found significant changes in the surface properties of tracheal fluid from dogs breathing 100% oxygen, and attributed the differing results to a variation in species sensitivity (11). Whether these findings resulted from the direct effects of oxygen on surfactant synthesis and secretion, or secondarily from other pulmonary damage, could not be ascertained. Chronic exposure to elevated concentrations of oxygen

results in capillary wall destruction, protein transudation into the alveolar lumen, and interstitial and alveolar edema (6). Certain constituents of edema fluid may be capable of interacting with pulmonary surfactant and altering its physical properties (35, 37).

Gecad and Massaro (9) have studied changes in protein synthesis associated with exposure to elevated oxygen. Statistically significant decreases in the rate of uptake and incorporation of amino acids could be demonstrated in slices taken from lungs exposed to 100% oxygen for 25 hours, and these changes appeared to be associated with a decrease in the intracellular volume of lamellar bodies in Type II cells (32). Consistent with these results are the findings of Brashear and Christian (4), who measured decreased amounts of phospholipid in the alveolar lavage fluid of rats exposed to 100% oxygen at 1 atmosphere. In none of these studies were the constituents of pulmonary surfactant specifically isolated and identified, and the results could reflect changes in constituents of the lung not associated with surface-active material. Morphological evidence is not confirmatory of early biochemical lesions in the Type II alveolar epithelial cell. Detectable damage first occurs in capillary endothelial cells, followed by a proliferation of Type II epithelial cells (1, 20).

The information presently available does not clearly indicate whether breathing oxygen at the concentrations and durations used by the United States Air Force and Navy aviators will affect the metabolism or function of pulmonary surface-active material. The possible changes in these activities need to be studied directly, utilizing methods capable of generating unambiguous results. Since the lung contains over 40 different cell types (21) carrying out different metabolic functions, it is difficult to discern whether significant changes have occurred in the metabolism of pulmonary surfactant if the experimental procedure only measures changes in lung constituents which may not be specifically found in this material. For instance, elevated concentrations of oxygen could conceivably increase protein synthesis in one cell type, while decreasing it in another. Such effects might be masked when studying the synthesis of the total protein contained in whole lung or in lung slices.

The work carried out under this contract has attempted to minimize these problems by the following approach:

(1) We have developed methods for isolating pulmonary surface-active material, analyzing its constituents, and identifying components which might be uniquely found only in surface-active material.

(2) We have devised techniques for quantifying surface-active material.

(3) We have developed methods for purifying enriched populations of the alveolar epithelial cells responsible for the synthesis of surface-active material.

(4) We have carried out a limited number of metabolic studies on these cells isolated from the lungs of normal animals in order to describe the rates at which they utilize O_2, and to identify the proteins into which radioactive amino acids are incorporated.

The results of these studies should provide the means by which we can quantitatively and specifically assess the metabolism of pulmonary surface-active material in normal animals. These methods can then be applied to animals who have been exposed to above-ambient concentrations

of oxygen for differing times, and their results can be used to evaluate the direct effects of oxygen exposure on metabolic activities associated with the cellular elaboration of pulmonary surfactant. The work required to develop these methods has been carried out under this contract. The results of these studies on normal rats are described in this report.

The objectives of the work carried out under this contract were:

(1) To develop methods which would be suitable for the quantification of pulmonary surfactant in rat lung.

(2) To develop methods for isolating populations of lung cells which are highly enriched in alveolar epithelial Type II cells.

(3) To provide a morphologic evaluation of these populations of alveolar epithelial Type II cells.

(4) To provide a metabolic characterization of cellular populations enriched in Type II cells.

METHODS AND RESULTS

Quantification of Pulmonary Surfactant in Rat Lung

Purification of Pulmonary Surfactant--We have previously developed methods suitable for the purification and characterization of pulmonary surfactant obtained from canine lung (24, 25). A slight modification of these methods was used to purify surfactant from rat lung, and the details of the procedure are similar to those which have been published previously (24).

Pathogen-free Wistar rats (Hilltop Lap Animals, Chatsworth, Calif.) of either sex, weighing about 300 to 400 g, were anesthetized by intraperitoneal injection of an overdose of sodium pentobarbital. A cannula was inserted into the trachea, and the chest was opened by a midline incision. Ringer solution (10 ml), buffered to pH 7.4 with 0.05M Tris-HCl, was instilled into the lungs with a syringe, and gently withdrawn. The procedure was repeated 5 times, or until the lavage fluid exhibited minimal opalescence. Pulmonary surfactant was purified from this lavage fluid by a combination of differential and density gradient centrifugation, as outlined in Figure 1. The lavage fluid was centrifuged at 150 x g for 5 minutes in order to remove cells. The supernatant was transferred to a #19 rotor (Beckman Instruments) and was centrifuged at 19,000 RPM for 12-16 hours. The clear supernatant was discarded and the precipitate was suspended in Ringer solution which had been supplemented with sodium bromide to increase its density to 1.12 g/ml (Ringer-NaBr). The suspension was centrifuged in a SW-27 swinging bucket rotor (Beckman Instruments) for 12 hours at 25,000 RPM. Three fractions were obtained: a floating white pellicle containing the surface-active material, a clear infranatant and a small amount of yellowish precipitate. The infranatant and precipitate were discarded. The pellicle was suspended in Ringer solution and pelleted by centrifugation for 2 hours at 30,000 RPM (#30 Rotor, Beckman Instruments). This precipitate was suspended in Ringer-NaBr at a density of 1.12 g/ml and poured in a continuous density gradient ranging from density 1.12 to 1.06. The continuous gradient was centrifuged for 14 hours at 25,000 RPM (SW-27 Rotor) to allow the surface-active material to migrate in the gradient to a position of isopycnic density. Practically all material could be found in one band at a measured density of about

1.08 g/ml. This band was recovered by aspiration, suspended in Ringer solution, and pelleted. The pellet was resuspended in 1.12 g/ml Ringer-NaBr, and centrifuged to obtain a floating pellicle. The pellicle was recovered, pelleted out of a water suspension, and dialyzed against water for 24 hours. Protein determinations, phosphorus determinations, polyacrylamide gel electrophoresis (39), and double-diffusion immunoprecipitation (26) were carried out on most fractions in order to characterize the lipid and protein properties. The protein content, based on dry weight, was 7.6% (SD 0.8) and phosphorus content was 3.3% (SD 0.3), giving an average protein to phosphorus ratio of 2.3. These protein and phosphorus contents are similar to those which have been found in canine surfactant (24).

The results of polyacrylamide gel electrophoresis of purified surfactant are shown in Figure 2. There are 3 major proteins: one migrating with a molecular weight of about 45,000 daltons; a second closely running band migrating with a nominal molecular weight of about 35,000 daltons; and a third protein which migrates in electrophoresis with a molecular weight of 10,000 to 12,000 daltons. In some preparations we also found variable amounts of a protein migrating with the same R_f as albumin. These results are similar to those found for surfactant from canine lung (23), electrophoresed under similar conditions, with one exception. Surfactant purified from canine lung contains one resolvable protein band migrating in the 35,000-45,000 dalton region, whereas surfactant from rat lung has two clearly identifiable bands of similar molecular weight. If sulfhydryl reducing reagents are omitted from the electrophoresis system, then the two bands migrating at 45,000 and 35,000 daltons disappear, and a new band is found at higher molecular weight of about 70,000 daltons. The results suggest that these two proteins may be subunits which are dissociated by sulfhydryl-reducing agents.

Antibody was developed in rabbits against pulmonary surfactant from rat washings using standard procedures (26). After adsorption with rat serum, this antibody gave a weak but detectable precipitation line with surfactant, but did not react with rat serum. Antibody to surfactant and antibody to rat serum (Cappel Laboratories, Downington, Pa.) reacted with pulmonary surfactant in a pattern of nonidentity. We conclude that the major proteins in surfactant are not proteins common to rat serum.

Isolation of the Apoproteins in Pulmonary Surfactant--The isolation of the 35,000-dalton apoprotein found in pulmonary surfactant was carried out according to a modification of previously published procedures (26). Ten to 15 mg of pulmonary surfactant were mixed with a 0.3 M solution of lithium diiodosalicylate (LIS) in 0.1 M tris buffer pH 8.0. The suspension was homogenized in a Dounce homogenizer (Kontes Glass) and was allowed to stand in ice for 30 minutes. Two ml of water and 4 ml of cold n-butanol were added, and the emulsion was mixed vigorously. Two phases could be formed after centrifugation in a clinical tabletop centrifuge. The upper alcohol-rich phase was removed. A volume of cold butanol equal to that of the lower phase was added, vigorously mixed, and the tube centrifuged to again separate phases. The upper phase was again removed. Similar procedures were followed after adding equal volumes of 6:1 (V/V) butanol/ethanol, and 6:1 (V/V) ether/ethanol; each alcohol mixture being used 2 to 3 times. The

procedure results in an extraction of lipids and lithium diiodosalicylate into the alcoholic upper phases. The bottom phase, containing the water-soluble proteins, was gently bubbled with nitrogen to remove ether and was utilized for further analyses by polyacrylamide gel electrophoresis, double-diffusion immunoprecipitation, and spectrometric scanning in the ultraviolet and visible spectra. A diagram of this procedure is shown in Figure 3.

After extraction with LIS and alcohols the 35,000- and 45,000-dalton apoproteins are found in the lower aqueous phase. A protein with a molecular weight of 69,000 daltons, probably albumin, is also sometimes found in the lower phase. In the past, the 10,000-dalton apoprotein was extracted into the alcoholic phase (26). This also probably occurred in the extraction of rat surfactant, but this phenomenon was not investigated directly. Removal of contaminating serum proteins was achieved by passing the aqueous-phase proteins through an affinity column containing immobilized antibody to rat serum. The IgG fraction of anti-rat serum was obtained from Cappel Laboratories (Downington, Pa.) and was coupled by cyanogen bromide activation to Sepharose-4B (Pharmacia Chemicals) using the procedure of March et al. (29). A 2-ml column was equilibrated with 10 ml of 1 M glycine in 0.03 M phosphate buffer pH 7.4 to block nonspecific binding sites and was thoroughly washed with 20 or more volumes of 0.03 M phosphate buffer; 0.5 to 1 ml of apoprotein solution containing 200 to 1000 µg protein was placed on the column and was eluted with the phosphate buffer which had been used previously to wash and equilibrate the column. Apoprotein was eluted in the void volume and was identified by its absorbance in the ultraviolet, by polyacrylamide gel electrophoresis, and by the immunological techniques described above. The eluate contained 2 proteins with nominal molecular weights of about 35,000 and 45,000 daltons. This material reacted with antibody to pulmonary surfactant, but it did not react to antibody developed against rat serum.

Development of a Radioimmunoassay--A radioimmunoassay was developed to measure the small amounts of surfactant apoprotein which might be expected in 1 to 2 x 10^6 Type II cells. The assay was patterned after methods which had been used previously to quantify small amounts of ovine surfactant (26). The technique is a modification of one developed by Salmon and coworkers (36) and is based upon the competitive binding for immunological binding sites adsorbed on a solid-phase matrix between apoprotein in a sample of pneumonocytes and ^{125}I-labeled apoprotein. The method is pictorially described in Figure 4. Pulmonary surfactant, suspended in 0.05 M Na borate at a concentration of 0.5 mg/ml, is added to polystyrene tubes and allowed to sit overnight at room temperature in order to adsorb to the walls of these tubes. The surfactant is removed and the tubes are incubated for 5 hours at 37°C with antibody developed against pulmonary surfactant (1:50 dilution with Ringer solution). Antibody is removed from the tubes, and the tubes are washed 3 times with distilled water. The resulting tubes have an adsorbed layer of antibody sites available for competitive binding assays.

The competitive-binding radioimmunoassay is carried out as follows: A "standard curve" is prepared by adding purified surfactant to the RIA tubes in amounts varying from 0.2 to 10 µg. All tubes are brought to an equal volume of 1 ml by dilution with 1:100 (V/V)

nonimmune serum buffered to pH 7.4 with 0.5 M phosphate. The tubes are incubated at 37°C overnight; 3000 counts of ^{125}I-labeled apoprotein (35,000-45,000 daltons) isolated from rat pulmonary surfactant are then added to each tube. Incubation at 37°C is continued for 4 more hours. The contents of the tubes are aspirated, and the tubes are washed 3 times with distilled water. The amount of labeled apoprotein which is bound to the tube is estimated by γ-particle scintillation counting. The details of the procedure are shown in Figure 5.

The quantification of the assay is dependent upon the competition between the apoprotein in the surfactant with the ^{125}I-labeled apoprotein for antibody sites adsorbed to the side of the tube. Samples which contain a large amount of antigen reduce the amount of labeled apoprotein which is bound to the tubes. A typical standard curve obtained by this procedure is shown in Figure 6. The results are expressed as the percent of the labeled apoprotein which is bound to the tube which contains no unlabeled competing antigen, as compared with the amount of bound apoprotein added to the tubes with varying amounts of competing antigen. The binding of labeled apoprotein decreases in an approximately linear manner when competing with samples containing 0.2 to 1 μg of surfactant, and the assay is most useful for samples containing antigen in this range. We tested the effects of adding serum to known amounts of antigen to see what interference might result from serum proteins. Adding serum in amounts up to 1 mg of protein had no notable effect on the results of the immunoassay. This amount of nonspecific serum protein represents a nearly 1000-fold excess over that of the antigenic protein and is far in excess of any amounts of serum protein that would be expected with any cellular sample. This assay was used to measure the amount of antigen in different populations of cells isolated from the lung. The results of these experiments are described in the section "Quantification of Apoprotein in Cellular Populations Separated by Density Gradient Centrifugation" (p. 13).

Isolation of Alveolar Epithelial Type II Cells

Current Methodology--The majority of effort expended under this contract was devoted to developing methods for isolating populations of cells which would be highly enriched in alveolar epithelial Type II cells. The methodology which we now utilize is the result of these efforts and is schematically shown in Figure 7.

We obtained Wistar rats from Hilltop Lab Animals (Chatsworth, Calif.) on a weekly basis in order to reduce the amount of time the rats are held in our Animal Care Facility and thus minimize the opportunity for respiratory infection. All animals which were utilized in this study appeared to have healthy lungs of good color, without noticeable abnormalities. We anesthetized the animals with sodium pentobarbital, 30 mg/kg administered intraperitoneally. A tracheal cannula was inserted and the animals were ventilated manually with air. The heart and lungs were exposed with a mid-line incision. The lungs were perfused with Minimal Essential Medium (MEM) supplemented with 0.07 mg/ml DNA-ase (Sigma Chemical Co., St. Louis, Mo., Type I) by inserting a cannula directly into the pulmonary artery through a

stab wound made in the right ventricle. Perfusions were carried out with a pressure of 10 cm H_2O. When the lungs had been visibly cleared of blood, they were quickly removed. Alveolar macrophages were partially removed by endobronchial lavage with Hank's solution without calcium and magnesium (3). The lungs were then filled with 5 mg/ml trypsin (Sigma, Type II) dissolved in MEM and diced with a small scissors into about 15 pieces. The lung sections were incubated in a water bath at 37°C for 3 min. Loose cells were removed by filtration through a 150 μ nylon mesh after 3 minutes, and the remaining tissue was covered with 5 mg/ml trypsin and reincubated for an additional 7 minutes. After a total of 10 minutes the lungs were diced finely with small sharp scissors and filtered through a graded series of nylon mesh filters of 150 μ-50 μ into 10 ml MEM supplemented with 10% fetal calf serum. These dissociated cells in MEM were added to a half volume of a sonicated dispersion of mineral oil and albumin (2 parts mineral oil and 1 part albumin, 5 mg/ml). The cells were mixed with the mineral oil dispersion and allowed to incubate at 37°C for 10 min. The cellular dispersion was centrifuged for 1500 RPM for 7 min in order to pellet cells from the mineral oil. The cells were suspended by very gentle mixing in 1 ml of MEM containing 0.07 mg/ml DNA-ase and carefully layered over a Ficoll gradient prepared with Ficoll in MEM with densities ranging from 1.01 to 1.04 g/ml. The cells were centrifuged at 1500 RPM for 3.5 min. Three bands were easily distinguished as shown in Figure 7. The first band, which is found toward the top of the centrifuge tube, principally contained small cells (lymphocytes) together with larger cells which appeared hydropic and blebed. The second band was comprised of about 75%-85% Type II cells, 5%-10% alveolar macrophages, and an equal amount of small cells. The third band consisted of alveolar macrophages and Type II cells in variable proportions. The results from twenty-one experiments, each consisting of cells purified from the lungs of two rats, were used to evaluate yields and purities. An average of 6.7 (SD 2.9) million cells were obtained from Band 2. These populations of cells had a mean concentration of Type II cells of 74% (SD 7%). Greater than 90% of the cells in all preparations excluded 0.4% trypan blue, and most preparations contained cells with greater than 95% viability. We have used the cells obtained by this method, therefore, for further evaluation of their morphology and their metabolic viability.

Investigation of Factors Affecting the Purification of Type II Cells--As part of the task in developing appropriate methods for isolating enriched populations of Type II cells, we investigated several of the variables which could conceivably affect the success of the procedure. Although most of these variables were not investigated systematically or extensively, in many cases we obtained some information which could bear upon the course of the further refinement of these methods.

a. Enzyme Concentration: The procedure which finally evolved utilized trypsin (Sigma, Type II) in a concentration of 5 mg/ml. We investigated the effects of using purified trypsin (Sigma, Type I) in concentrations of 1, 2, and 3 mg/ml, and impure trypsin (Sigma, Type II) in concentrations up to 10 mg/ml. With all enzymes comparative experiments were done using a 10-minute incubation at 37°C. Yields of total cells (before density gradient centrifugation) using purified trypsin ranged

from 0.4 to 5 million cells per lung (1 mg/ml), 6.6 million (2 mg/ml), and 12.4 million (3 mg/ml). Using 5 mg/ml of impure trypsin we generally obtained about 40 million cells per lung. Thus we found that using lower concentrations of purified trypsin generally resulted in lower yields of dispersed cells. However, increasing impure trypsin concentrations (Sigma, Type II) up to 10 mg/ml did not markedly improve cellular yields and sometimes resulted in a greater percentage of cells taking up vital stains.

b. Method of administration of enzymes: We administered enzymes by filling the lung through the airways to total lung volume. A few experiments were carried out to determine the effects of administration of enzymes through the pulmonary circulation. The lungs of the animals were first perfused with MEM as described in the section "Current Methodology" (p. 8). The reservoir containing the perfusing medium was then exchanged with a solution of trypsin or the enzyme under study and the pressure head was lowered to 3 cm H_2O to effect a very slow perfusion under low pressure. Perfusion with enzyme was carried out over a course of 30-40 minutes while the animal was continuously ventilated with air. In most cases evidence of cellular dispersion could be noted within about 15 minutes after beginning the enzyme perfusion. By the end of the perfusion (generally 30-40 minutes) the lungs were unable to hold air. The lungs were immediately diced with a small scissors and filtered through graded nylon filters from 150 μ through 50 μ. The cells were then separated on density gradients and tested for their viabilities by trypan exclusion.

The following enzymes were administered by perfusion: trypsin (Sigma, Type II) 10 mg/ml; collagenase (Worthington, Type I) 2 mg/ml; and collagenase (Worthington, Type IV) 2 mg/ml. The results of all experiments were considered to be unsatisfactory. Yields of total cells were lower than those obtained by administration of enzymes through the airway and varied from 8 to 16 million per rat. Viabilities, as tested by trypan blue exclusion, ranged from 30% to 60% viable cells. Contrast staining and electron microscopy showed extensive damage of plasma and intracellular membranes. Mitochondria were hydropic and disorganized. We concluded that the dissociation of the lungs by the perfusion of enzymes could be readily accomplished, but that the condition of the free cells was worse than those obtained by administration of enzymes through the airway.

c. Time of incubation of the lung with enzyme: Ten minutes of incubation at 37°C was used for most experiments. We investigated the effects of increasing times up through 20 minutes using impure trypsin (Type II). In general, increasing the time of incubation only modestly increased the yield of dispersed cells. Viability (trypan blue exclusion) decreased with longer periods of incubation. We decided, therefore, that a time of incubation of 10 minutes provided a suitable balance between yields and viabilities.

d. Effects of incubating the cells in a mineral oil dispersion: Immediately after obtaining our total cell dispersion we suspended these cells in a mineral oil/albumin dispersion and incubated them for 10 minutes at 37°C. The rationale for utilizing this procedure was the expectation that alveolar macrophages would take up mineral oil and therefore be separated away by density gradient centrifugation from the heavier, nonphagocytic cells. The results did not verify

this expectation. Alveolar macrophages were generally recovered within the gradient at densities somewhat greater than those obtained in Type II cells, and there was no evidence of significant cellular inclusion of the mineral oil dispersion. We did note, however, that if the incubation in the mineral oil dispersion was omitted we obtained a greater percentage of small cells within our Type II band than generally occurred when the mineral oil dispersion step was carried out. We concluded that, although all alveolar macrophages may not have taken up this dispersion, other cells may have been coated with dispersion changing their migration in the density gradient. This may have helped in effecting the separation between the Type II cells and the lymphocytes. Therefore, this step was included in all subsequent procedures.

e. Suitability of including DNA-ase in all solutions: All solutions used for the separation procedure contained DNA-ase I (Sigma, crude beef pancreas) in a concentration of 0.07 mg/ml. Omitting DNA-ase from these solutions resulted in either: (1) an increase in the amount of mechanical agitation required to resuspend pelleted cells, (2) the clumping of cells during density gradient centrifugation. We concluded that DNA-ase should be used in all steps of the procedure.

f. Types of density gradients: We separated cells on density gradients prepared with Ficoll dissolved in MEM. Cells were layered over pre-formed gradients continuous in density from 1.01 g/ml to 1.04 g/ml. Our initial experiments investigated the suitability of using discontinuous density gradients, such as those used by Kikkawa and coworkers (21, 22) and Mason and coworkers (30, 31). We found that, in our hands, the discrimination between cell types was far less satisfactory using discontinuous density gradients than was obtained using continuous gradients. Discontinuous gradients were prepared by the sequential layering of Ficoll solutions of densities 1.03, 1.04, 1.05, and 1.08 g/ml; 3 ml of each of these solutions was used in a SW-27 centrifuge tube, providing a filling of the tube to approximately seven-tenths of total volume. Cells in MEM were then carefully layered over the density gradient. The gradients were centrifuged at speeds ranging from 1500 RPM to 3500 RPM, and with times varying from 10 minutes to 45 minutes. The results differed depending upon the details of the procedure, but we were never able to recover cells from any of the discontinuous interfaces which contained Type II cells in greater than 50%-60% purity. Results were difficult to reproduce. Few intact cells were found at the 1.03 interface. Material at this interface was sparce and generally consisted of debris. Cells at the 1.04 interface generally appeared hydropic, stained weakly with contrast stain, and appeared damaged when examined by electron microscopy. Cells at the 1.05 interface appeared healthy, but consisted of a mixture of small cells such as lymphocytes together with medium-sized cells and beating ciliated cells. Cells at the 1.08 interface contained larger cells, ciliated cells, and some medium-sized cells probably including Type II cells. The densities of the discontinuous gradients were varied in attempts to improve yields and purities but these results were also unsuccessful. Discontinuous gradients prepared with solutions of densities of 1.036, 1.047, 1.058, 1.072, and 1.082 g/ml provided somewhat greater discrimination among cells of different size, but never to the degree that was achieved using continuous gradients. After nearly 90 experiments the use of discontinuous gradients was abandoned.

g. Dispersion of the lung using collagenases: Trypsin was found to be capable of dispersing the lung and was partially specific for dissociating alveolar epithelial cells. The suitability of collagenases to dissociate the lung was also investigated in a limited number of experiments. Collagenases of Types I, II, and IV were received as gifts from the Worthington Corporation. All collagenases were used in a concentration of 2 mg/ml dissolved in Hank's solution, and were administered through the airway. Cells were separated on continuous density gradients prepared with Ficoll in MEM with densities varying from 1.01 to 1.04 g/ml. The band normally containing enriched populations of Type II cells was removed for evaluation by contrast staining and electron microscopy. The yields were somewhat lower than those obtained using trypsin, and only 1 to 1.5 million cells were recovered from the appropriate band in the density gradient. Viabilities, as measured by trypan blue exclusion, were generally worse than those found for cells dissociated with trypsin, and only 60% to 70% of the cells excluded dye. No band on the density gradient could be recovered which contained Type II cells in concentrations greater than 50%. We suspect, but did not verify, that collagenase is more effective in dissociating interstitial cells rather than epithelial cells, leading to a far more difficult problem in purifying Type II cells from the total cellular dispersion. Since we did not find that the condition of the cells was improved after dissociation with collagenases (rather the opposite), we did not further evaluate the possible advantages of these enzymes.

h. Use of glass beads or nylon fibers to selectively remove adsorbent cells: Certain populations of lymphocytes and polymorphonuclear leukocytes had been reported to adsorb on glass beads (40) or nylon fibers (28) or glassware (19). We carried out six experiments investigating the suitability of passing the populations of cells dispersed from the lung over glass beads or nylon fibers to remove lymphocytes. In two experiments cells in MEM were incubated for 30 minutes at 37°C with 3-mm glass beads. The beads were then allowed to settle and the cells in the supernatant were removed. The percentage of Type II cells before and after incubation with glass beads was unchanged. Similar procedures were carried out using nylon fibers. Cells in MEM were passed over a 2-ml column filled with nylon fibers in MEM (Leukopak, Fenwal Labs). Recovery of the cells from the column was low and we were unable to effect further purification of our cellular population. The results obtained from these limited number of experiments were not encouraging. We did not further investigate, therefore, the possibility that cell types obtained from the lung might be separated by adsorption on nylon or glass fibers.

i. Methods used for identification of cell types: We used the following methods of staining for routine identification of cell types: Wright-Giemsa stain, fluorescent stain phosphine 3R (30), hematoxylin-eosin stain, and a modified Papanicolaou stain (21). Cell preparations were occasionally fixed and stained for electron microscopy, as described in the section "Morphological Characterization of Cellular Populations Enriched in Type II Cells" (p. 13). Staining with Wright-Giemsa or hematoxylin-eosin stains was generally satisfactory for identifying known blood types or for discriminating cells according to size. The fluorescent dye phosphine 3R was taken up by Type II cells and appeared as bright subcellular inclusions. It was also taken up by alveolar macrophages, however, making it difficult to utilize this dye to discriminate Type II cells from macrophages. The Papanicolaou stain, as modified

by Kikkawa and coworkers (21), was specific for Type II cells and could easily discriminate Type II cells from macrophages and blood cells. Both Type II cells and alveolar macrophages stained with dark inclusions. Type II cells could be easily distinguished from alveolar macrophages by the size of the inclusions, the color and intensity of staining of the cytoplasm, and cell size. The reliability of Papanicolaou staining was verified by electron microscopy. We routinely incorporate this stain in our evaluation of all preparations.

Morphological Characterization of Cellular Populations Enriched in Type II Cells

Three preparations of the cells which were isolated on Ficoll density gradients as described in the section "Current Methodology" (p. 8) were fixed and stained for electron microscopy. Cells were removed from the Ficoll density gradients and immediately mixed with an equal volume of a 5% solution of glutaraldehyde buffered with 0.2 M sodium cacodylate at pH 7.4. Fixation in glutaraldehyde was carried out overnight at 5°C. The cells were pelleted out of the glutaraldehyde solution and suspended in 1% osmium tetroxide also buffered with sodium cacodylate to pH 7.4. Fixation continued at 5°C for 24 hr. The cells were then stained en bloc with cold uranyl acetate and dehydrated with a graded series of acetone solutions. Embedding of the cells was carried out in Spurr's plastic for sectioning with an ultramicrotome. A representative field is shown in Figure 8.

Several high- and low-powered fields obtained from these 3 preparations were examined by electron microscopy. Eighty to 90% of the cells in these fields were identified as Type II alveolar epithelial cells. Most cells had a morphologic appearance suggesting normal metabolic function and good viability. Occasional cells exhibited slight mitochondrial swelling or disorganization. In general, it was judged that the morphology of these cells was typical of that of Type II cells found in sections of normal lung.

Quantification of Apoprotein in Cellular Populations Separated by Density Gradient Centrifugation

We used the radioimmunoassay described in the section "Development of a Radioimmunoassay" (p. 7) to quantify the amount of apoprotein in cells isolated by the procedures outlined in the section "Current Methodology" (p. 8). We tested the following cellular populations for their content of surfactant apoproteins: (1) cells comprising the total dispersal after enzymatic dissociation of the lungs; (2) cells recovered from the endobronchial lavage fluid of the lung, principally alveolar macrophages; (3) cells found in the three bands separated by Ficoll density gradient centrifugation, as shown in Figure 7. Cells recovered from these density gradients were washed two times in a 1:100 dilution of nonimmune rabbit serum in Hank's solution containing calcium and magnesium. Cells obtained from the alveolar wash fluid or from the total dispersal were washed 6 times

in this solution. The cells were then suspended in 0.5 ml of 1:100 rabbit serum in Hank's solution and homogenized with a small hand-held homogenizer. The homogenates were diluted to 1 ml with 1:100 rabbit serum and rehomogenized with a power-driven homogenizer (Tekmar Model SDT). Aliquots of these cells were then placed in tubes coated with immobilized antibody and were incubated overnight at 37°C in a water bath. Apoprotein content was quantified by the competitive binding of a known amount of radioactive-labeled apoprotein standard and compared with the binding of labeled apoprotein in a standard curve prepared with purified surfactant. Apoprotein content was expressed as micrograms of apoprotein contained in 1 million cells. The results are shown in Table 1. Cellular populations highly enriched in Type II, such as cells in Band 2 obtained from the gradient, contained about 0.4 to 0.5 µg of apoprotein per million cells. Cells which did not contain Type II cells (Band 1 in the gradient) contained very little apoprotein. These results are consistent with the expectation that Type II cells synthesize pulmonary surfactant and its associated apoproteins. We also found, however, that alveolar macrophages (cells from the alveolar lavage fluid) also contained apoprotein in concentrations equal to or even greater than that found in cellular populations enriched in Type II cells. Cells from the alveolar wash contained an average of 0.9 µg of apoprotein per million cells but with a wide distribution in content among individual experiments. We attempted to see whether some of this apoprotein came from acellular pulmonary surfactant that was still carried along in the cellular washes. We measured the amount of pulmonary surfactant in the wash fluid after the 5th and 6th final washes of the alveolar macrophages. Content of apoprotein in these fluids was negligible. We conclude that alveolar macrophages and Type II cells both have pulmonary surfactant. Preliminary data indicate that only the Type II cells synthesize this apoprotein (see section "Metabolic Utilization of Amino Acids to Synthesize Proteins" (p. 15)). Alveolar macrophages may ingest surfactant and provide a pathway for clearance. A possibility that cannot be excluded, however, is that the pulmonary surfactant is simply adsorbed on the surface of macrophages and is not removed by the 6 washes used to prepare these cells for assay.

Metabolic Characterization of Cellular Populations Enriched in Type II Cells

Consumption of Oxygen--The oxygen consumption of cellular populations enriched in Type II cells was measured in 4 preparations. Cells were obtained from the Ficoll density gradient as described in the section "Current Methodology" (p. 8). Cells were removed from the gradient and allowed to equilibrate with 5% CO_2/95% O_2 at 37°C in the water bath of the device used for measuring oxygen consumption (Yellow Springs Instruments, Model 53). The cells were transferred to the measuring chamber of the oxygen consumption apparatus, and their consumption of oxygen was measured using a high-sensitivity membrane. The results of these experiments were generally characterized by two apparent rates of consumption. A very fast initial rate was observed upon beginning stirring which generally lasted from 1 to 2 minutes. There followed a second, somewhat slower rate which was characterized

by an uptake of oxygen which was linear with time over the next 30 minutes. This second rate was used for calculating the rates of oxygen consumption. Mean oxygen consumption was 82.8 nmoles of oxygen per hour million cells (SD 30.2) in the 4 preparations which were examined. Most of these preparations contained nearly 80% Type II cells. The contamination with alveolar macrophages was about 10%. Alveolar macrophages had been reported to utilize oxygen at very high rates, possibly as much as 150 nmoles of oxygen per hour per 10^6 cells (13). The rates actually utilized by the Type II cells in the preparation might be somewhat lower, therefore, than those actually measured for the total mixture. We concluded, however, that the cells utilized oxygen at rates comparable to those expected for viable, healthy cells in an active metabolic state.

In two preparations we attempted to discern whether the cells were partially uncoupled and therefore consuming oxygen at rates greater than would be expected for normal cells. We added to these preparations 10^{-6} molar trifluoromethoxycarbonyl cyanide phenyhydrazone (FCCP) in a very small volume of ethanol and observed rates of oxygen consumption after addition of this uncoupling agent of oxidative phosphorylation. Oxygen consumption increased by about 10% after the addition of FCCP. Addition of greater amounts of FCCP generally led to lower rates of oxygen consumption and ultimately to a complete cessation of oxygen utilization.

Metabolic Utilization of Amino Acids to Synthesize Proteins--We have carried out a limited number of studies investigating the incorporation of radioactive leucine into proteins synthesized by isolated cells. The procedure which was utilized is as follows: Cells enriched in Type II cells were obtained from the density gradient and immediately pelleted by centrifugation at 1500 RPM for 10 minutes. The cells were suspended in MEM not containing leucine and gently agitated in a slowly shaking water bath at 37°C; 18.75 μCi of ^{14}C-leucine (spec. act. 0.31 mCi/mM) were added to about 5 million cells, and the cells were incubated at 37°C for 4 hrs. After 4 hrs, we added about 1 mCi of ^{3}H-leucine (spec. act. 2 Ci/mM) and took equal samples of cells immediately after addition, 30 minutes after addition, 60 minutes after addition, and 90 minutes after addition of the ^{3}H-leucine. The cells were immediately homogenized with an aliquot of purified surfactant and carried through the purification procedure usually utilized to purify surfactant from cell or tissue samples (24). The material isolated by this procedure would be expected to contain surfactant contained within the cells together with the carrier surfactant which was added as an aid in the purification. Aliquots of this purified material were then dissolved in 100 μL of a solution containing 0.3 M lithium diiodosalicylate and 2% sodium dodecylsulfate. Proteins in these preparations were separated by polyacrylamide gel electrophoresis (26). The bands containing apoprotein to pulmonary surfactant were cut from the gel, and the protein in these bands was extracted by homogenization of the cut gels in 2% sodium dodecylsulfate. Extracted protein solutions were counted in a β-scintillation spectrometer to estimate the incorporation of ^{14}C- and ^{3}H-leucine into the respective proteins.

This double-isotope method was used to provide a sensitive method of looking at the rate of synthesis of the small amounts of protein which

might be synthesized by the isolated cells. After 4 hrs we would expect that the apoproteins in surfactant should be labeled with ^{14}C-leucine at a uniform level, and that the measurement of ^{14}C-activity in these proteins should be proportional to the amount of protein. The incorporation of ^{3}H-leucine into these proteins in the following 90 minutes would indicate the rate at which this isotope is utilized. We evaluate the data, therefore, by looking at the ^{3}H and ^{14}C ratios as a measure of specific activity. The results are shown in Figure 9. Over the 90-minute period there was a four-fold increase in the ^{3}H-leucine into the 35,000-dalton apoprotein. There was also incorporation of precursor into the 10,000-dalton apoprotein, but apparently at a slower rate than that found for the 35,000-dalton apoprotein. These results suggest that Type II cells are able to incorporate leucine into at least 2 proteins which can be isolated with pulmonary surfactant: one with a molecular weight of about 35,000 daltons, the other with a molecular weight of about 10,000 daltons. Additional experiments are required to verify these results and to determine the reliability of this experimental procedure.

We also carried out a limited number of experiments looking at the facility by which ^{14}C-acetic anhydride might be used to label intracellular proteins in Type II cells. Acetic anhydride has been used to quickly label cells grown in culture (34), and we have been guided by these procedures. ^{14}C-acetic anhydride has been shown to enter intact cells and to acetylate amino groups on intracellular proteins. We did two experiments attempting to label intact cells with acetic anhydride, but both were unsatisfactory. Acetic anhydride was presented to the cells either dissolved in dimethylsulfoxide or in a small amount of benzene. We then isolated intracellular proteins by polyacrylamide gel electrophoresis. In both experiments, we did not get sufficient incorporation of ^{14}C label into proteins to make this procedure a potentially useful method.

In a limited number of experiments which are presently being continued we injected 2.3 mCi of ^{3}H-leucine (spec. act. 2 Ci/mM) into the jugular veins of rats and allowed the rats to incorporate tritiated leucine into the proteins of Type II cells while the animals were alive but still under anesthesia. At one hour after injection we killed the animals and isolated Type II cells using procedures as described. Type II cells were solubilized in a solution of 0.3 M lithium diiodosalicylate and 2% sodium dodecyl sulfate and their proteins were separated by polyacrylamide gel electrophoresis. The gels were then sliced into approximately 1-mm segments which were counted for the incorporation of tritiated leucine into protein. The results of one of our first experiments are shown in Figure 10. Leucine was incorporated by Type II cells into a protein migrating with the same molecular weight as that found in the apoproteins associated with pulmonary surfactant. In contrast, alveolar macrophages from the same experiment had a lower level of incorporation of leucine, and there were no proteins which incorporated leucine at particularly accelerated rates. These preliminary results suggest that alveolar macrophages do not synthesize the apoprotein of pulmonary surfactant at rates that can be easily discernible 1 hour after administration of the labeled precursor. In contrast, this limited evidence indicates that the Type II cells do carry out this synthesis. We are continuing these experiments to verify these results. We will also evaluate whether metabolic function of these cells would be best studied using a protocol in which

the precursor is presented to isolated cells, in vitro, or whether in vivo presentation might be more suitable. These results will enable us to appropriately design experimental conditions to investigate the effects of above-ambient concentrations of oxygen on metabolic activities of these cells and to describe the cellular damage sustained by such environments.

CONCLUSIONS

We have developed methods to purify pulmonary surfactant from rat lung, identify certain of its apoproteins, and measure apoprotein content in isolated populations of rat pneumonocytes. We have been reasonably successful in developing techniques for dispersing the lung into constituent cells, and in purifying populations of cells enriched in Type II alveolar epithelial cells. We have used these methods to quantify the amounts of apoprotein in these cells and, to a limited extent, to describe some of the metabolic functions of these cells. We have not yet applied this methodology to cells obtained from rats exposed to above-ambient concentrations of oxygen. We expect, however, that this work will provide the basis for carrying out such experiments.

Isolation of Surfactant

The procedures used to isolate surfactant from canine lung appear to be easily adapted to the isolation of surfactant from rat lung. The contents of protein and phosphorus in rat lung are similar to that found in canine lung (25), as are the molecular weights of the apoproteins. Detailed analyses of lipid composition have not been undertaken, but such information is available (12). These results, together with other findings (23), indicate that the pulmonary surfactants derived from several different species are similar in lipid and protein composition.

Quantification of Apoprotein

We used a solid-phase radioimmunoassay to quantify surfactant apoprotein in isolated pneumonocytes. The assay is sensitive and specific, but shares with all radioimmunoassays the problems of developing specific antibodies and of isolating and radioactively labeling pure antigens. It is subject to variation because of differences in antisera titers, degradation or denaturation of the labeled antigen, and nonuniformity in the coupling of antibody to the solid phase. For this reason, therefore, a complete standard curve is run in triplicate for every assay. Three dilutions of every sample, each in triplicate, are used for the determination of apoprotein content. The procedure cannot be considered as a technique that can be readily used on a routine basis. The development of simpler procedures to quantify apoprotein would be a highly desirable goal, if these techniques could also retain the positive features of sensitivity and specificity found with the radioimmunoassay.

Isolation of Alveolar Type II Cells

We are able to routinely isolate alveolar epithelial Type II cells with purities of 75% to 85%. The cells exclude trypan blue, consume oxygen at reasonable rates, and incorporate radioactive leucine into proteins associated with pulmonary surfactant. Yields generally run from 2 to 4 million cells per rat lung.

The methods that we employ to separate Type II cells from other dispersed pneumonocytes give cellular populations sufficiently enriched in Type II cells for metabolic experiments. Yields are relatively low, but are satisfactory if sensitive techniques are used for the analyses. The technique used to disperse the lung into constituent cells is potentially damaging. Relatively high concentrations of trypsin are used, and it would be highly desirable to be able to lower enzyme concentrations or to vary enzyme types (14, 15) in order to reduce possible trauma and improve yields. The use of primary cultures may be advantageous. Although we feel that our procedures are now adequate for isolating Type II cells and for carrying out metabolic experiments, they are probably not optimal. Significant improvements, however, may be difficult and may require considerably greater effort than has been expended to date.

ACKNOWLEDGMENTS

Dr. Kenneth G. Gould, Jr. gave us valuable advice on cell dispersion and separation techniques and provided detailed morphological evaluations of isolated cells. Mr. Patrick J. Lynch, III, ably assisted in all aspects of this work. Mrs. Helen Martin participated in some of the latter experiments, principally those studying the cellular metabolism of proteins. Salary support for Mrs. Martin was provided through a grant from the National Heart, Lung and Blood Institute, HL 16725.

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TABLE 1. SURFACTANT APOPROTEIN IN ISOLATED CELL POPULATIONS

Cell population	Composition (%)		Apoprotein conc. ($\mu g/10^6$ cells)
	Macrophage	Type II	
Alveolar wash	100	0	0.9(0.4-2)
Total dispersal	25 (22-29)	53 (52-54)	0.6(0.5-0.7)
Band 1	7 (1-13)	14 (4-23)	0.1(0-0.2)
Band 2	8 (4-11)	74 (64-86)	0.4(0.1-0.5)
Band 3	50 (48-53)	36 (32-41)	0.4(0.3-0.5)

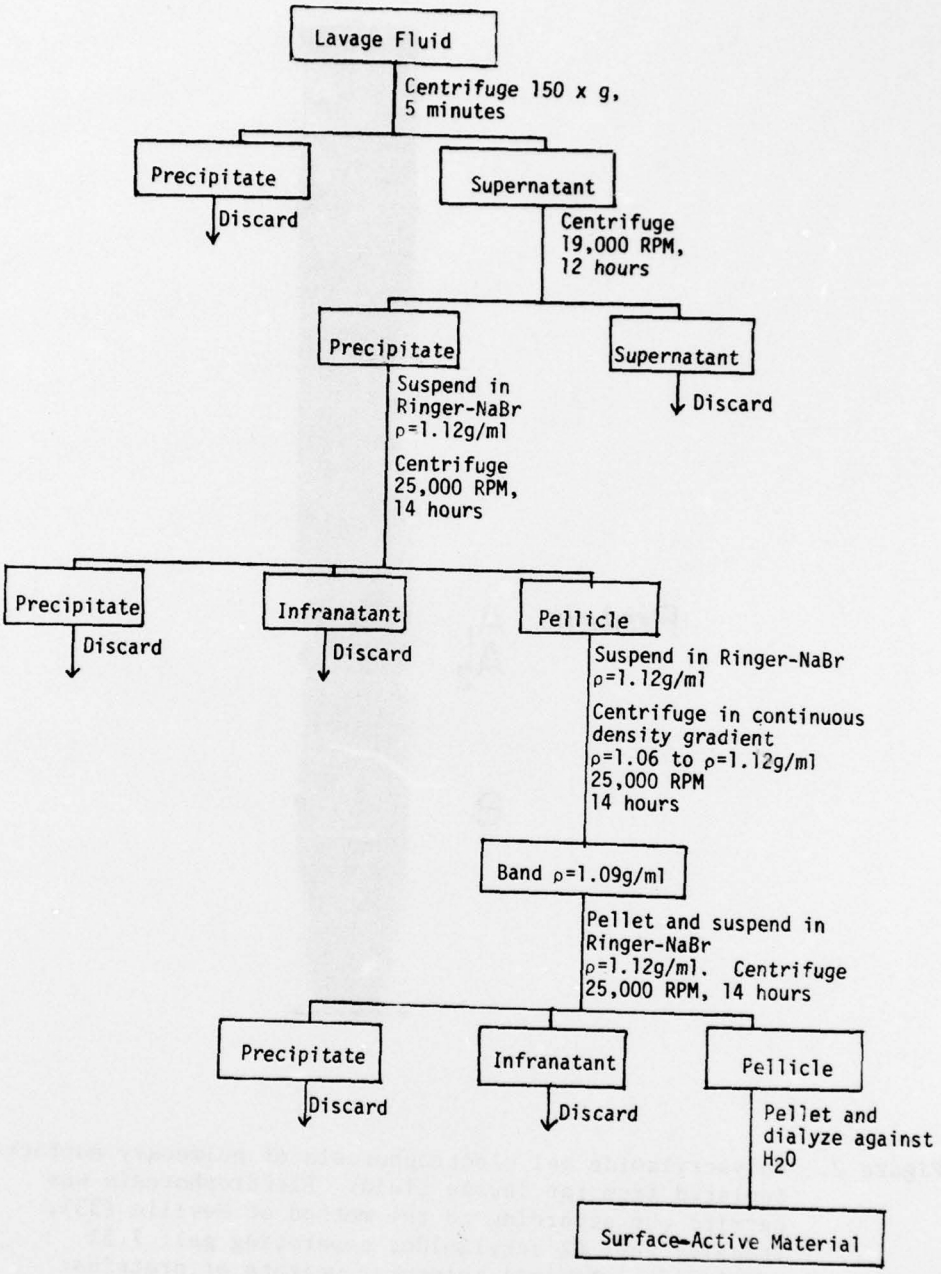

Figure 1. Isolation of surface-active material in lavage fluid from rat lungs.

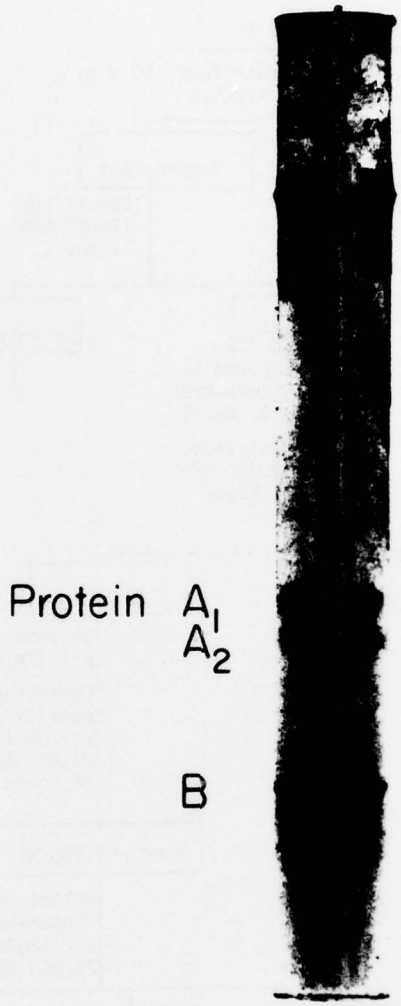

Figure 2. Polyacrylamide gel electrophoresis of pulmonary surfactant isolated from rat lavage fluid. Electrophoresis was carried out according to the method of Neville (33). Stacking gel: 4% acrylamide; separating gel: 7.5% acrylamide. Nominal molecular weights of proteins: Protein A_1, 45,000 daltons; Protein A_2, 35,000 daltons; Protein B, 10,000 to 12,000 daltons. The dark area seen below protein B is a staining artifact seen in all gels, including purified molecular weight standards.

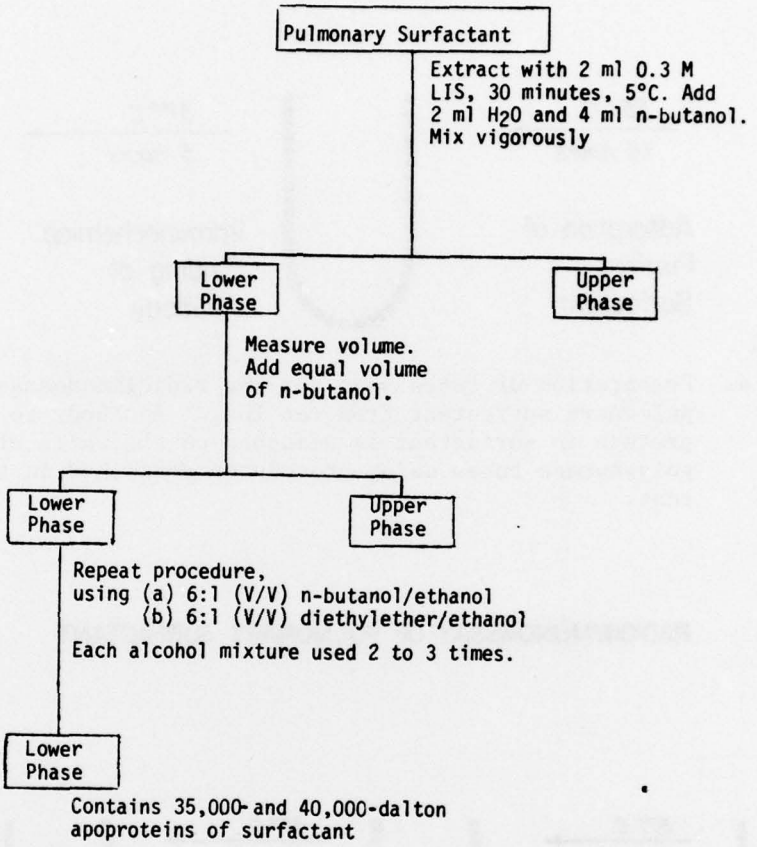

Figure 3. Extraction and isolation of 35,000- and 40,000-dalton apoproteins of surfactant.

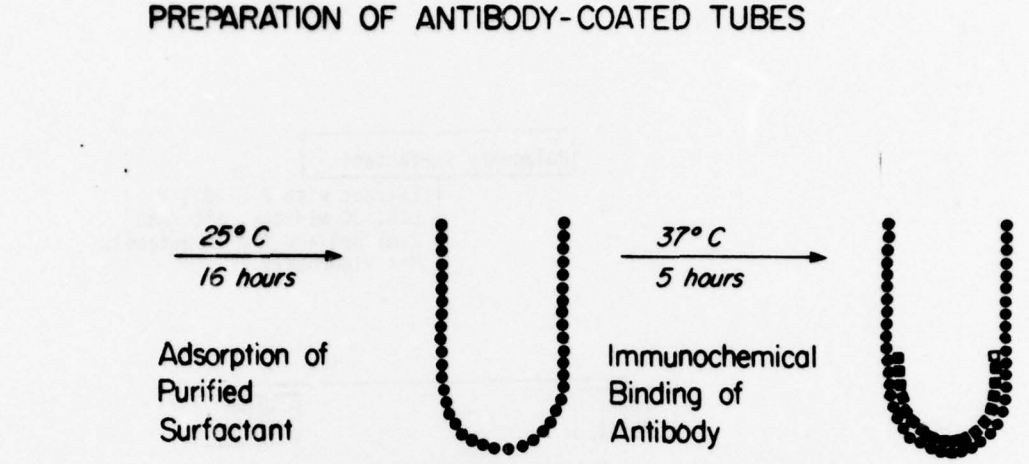

Figure 4. Preparation of tubes used for the radioimmunoassay of pulmonary surfactant from rat lung. Antibody to apoprotein in surfactant is adsorbed on the walls of polystyrene tubes using procedures described in the text.

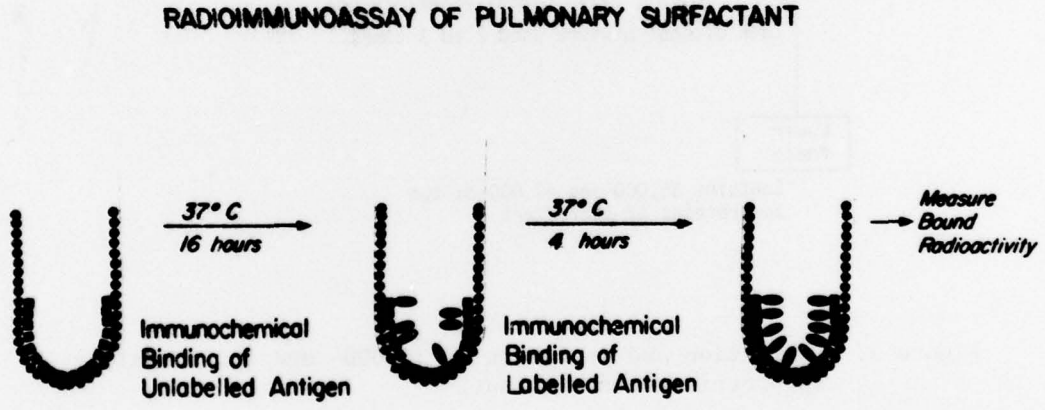

Figure 5. Procedure used to quantify surfactant by solid-phase radioimmunoassay. Details are given in the text.

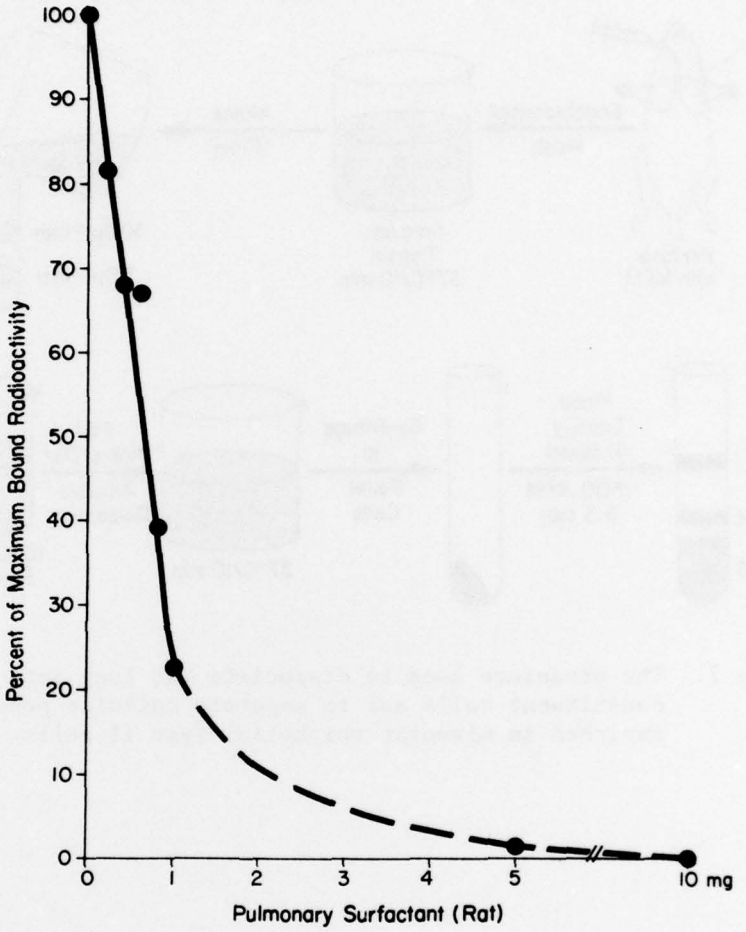

Figure 6. A typical standard curve of purified pulmonary surfactant from rat lung used to calibrate the radioimmunoassay.

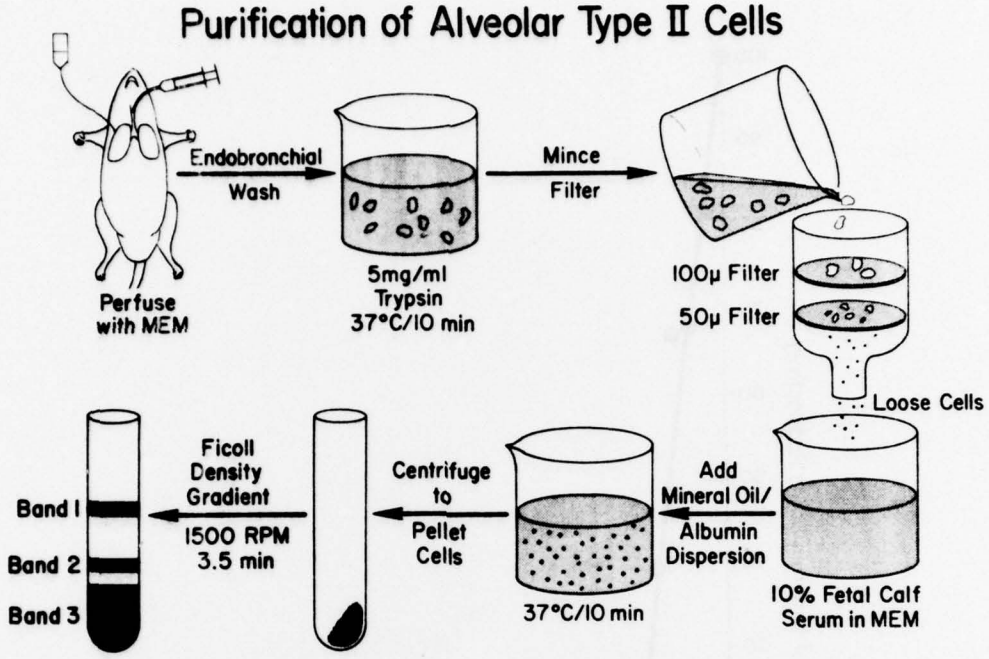

Figure 7. The procedure used to dissociate rat lung into constituent cells and to separate cellular populations enriched in alveolar epithelial Type II cells.

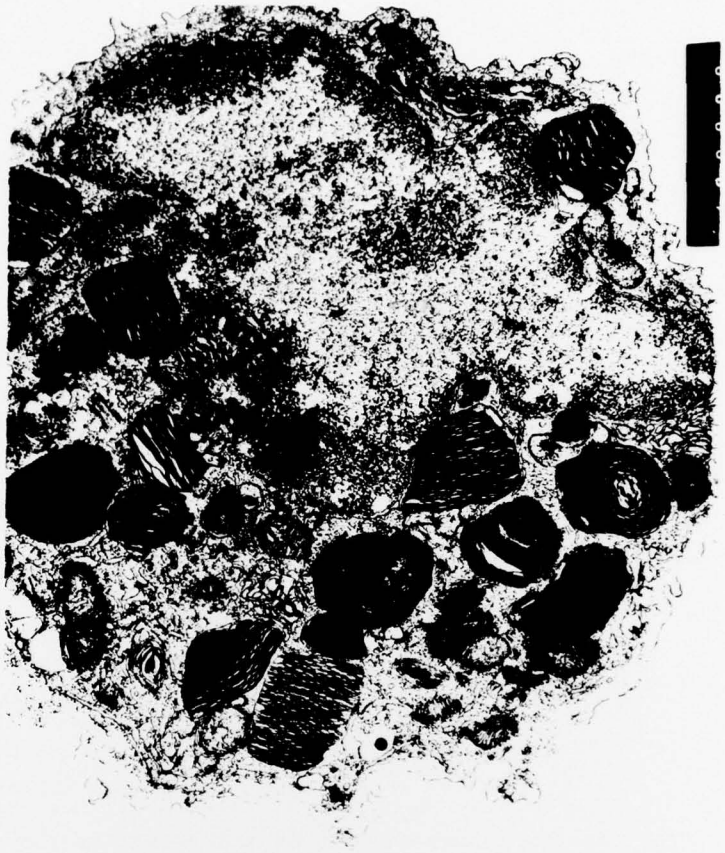

Figure 8. Electron micrograph of an alveolar epithelial Type II cell obtained from a cellular population purified by density gradient centrifugation. Fixation and staining methods are described in the text. Magnification: 16,300X

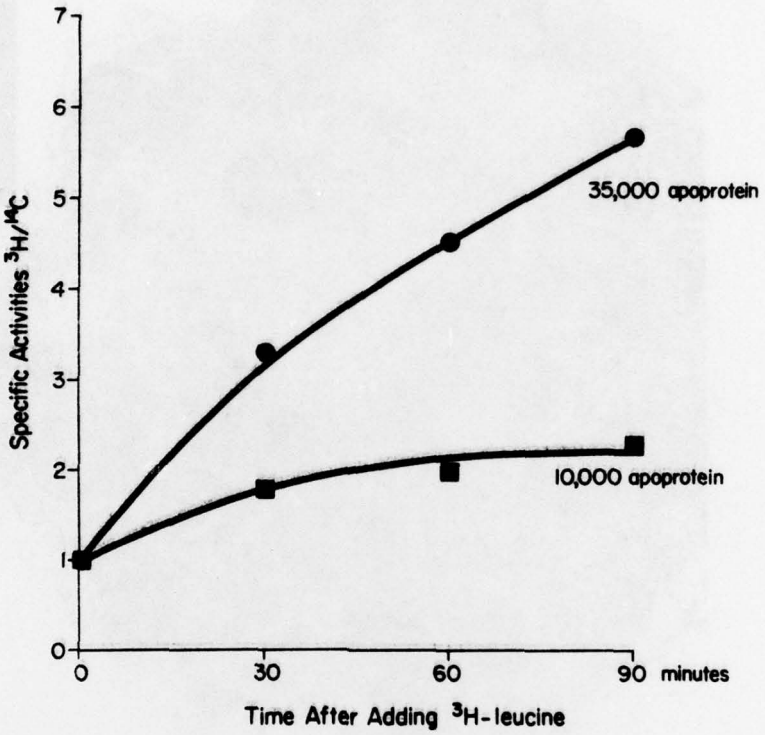

Figure 9. The results of one experiment investigating the ability of isolated Type II cells to incorporate ^3H-leucine into the proteins associated with purified surfactant. Isolated cells in Minimal Essential Medium were incubated at 37°C with ^{14}C-leucine for 4 hours to approximate a uniform labeling of all proteins with ^{14}C-leucine. ^3H-leucine was added to the incubation medium, and aliquots of cells were then taken at selected time intervals after addition of the tritium label. Surfactant in cells was purified by centrifugation, and its associated proteins were separated by polyacrylamide gel electrophoresis. Specific activities of the 35,000- and 10,000-dalton apoproteins were estimated by the ratio of ^3H-leucine/^{14}C-leucine incorporated into the proteins.

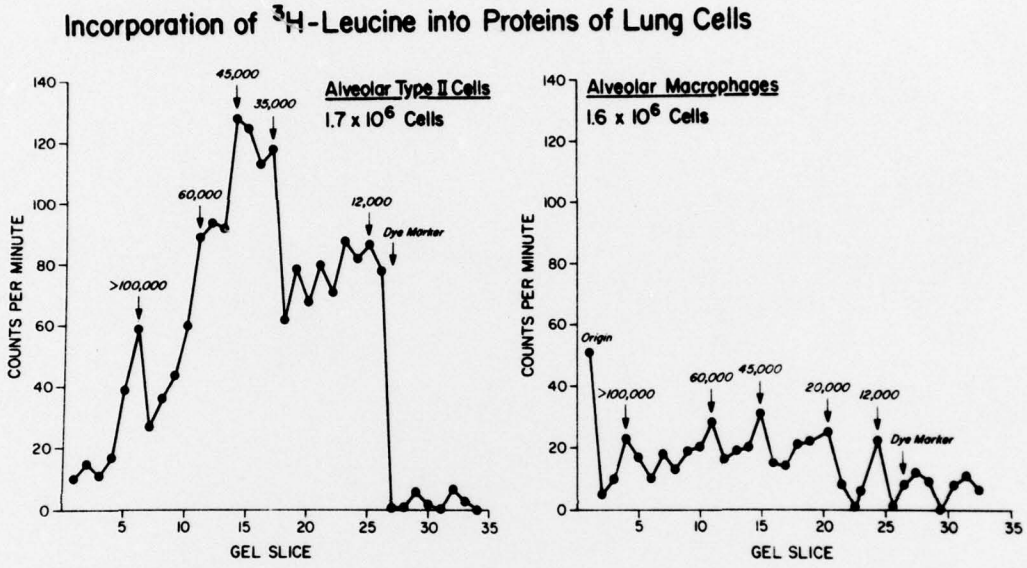

Figure 10. The results of one experiment describing the incorporation of ^{3}H-leucine into alveolar macrophages and alveolar epithelial Type II cells. ^{3}H-leucine was injected into a rat 60 minutes before sacrifice. Macrophages and Type II cells were isolated by procedures described in the text. Cells were homogenized and their proteins separated by polyacrylamide gel electrophoresis. Gels were sliced in 2-mm segments and were counted for tritium activity. Approximate molecular weights of certain protein constituents are shown on the figure.